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Biomarkers in the Detection of Human Heritable and Germinal Mutagenesis

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An important potential use of biomarkers in human toxicology is the detection of induced mutational events in offspring and germ cells of exposed individuals. The importance, of course, is in risk estimation and the identification and prevention of exposure conditions that are harmful to the human genome. The challenge is to discover methods of sufficient power to find the rare, random, mutational events and to discriminate such events from other sources of molecular variation. Finding mutations is essentially a search for disorder. Normal biomarkers are inherently unsuitable in a positive search for disorder; instead one must either use abnormal markers or be prepared to search negatively, i.e., to look for and somehow validate the rare absence of a normal marker. In spite of these difficulties, there is progress to report and hope of future success in this field.

Background

The major and most promising setting for observing an induced mutation rate in a human population is among the offspring of A-bomb survivors in Japan. Ionizing radiation is a consistent mutagen in all living organisms, and the human should be no exception. A sizeable body of data in the mouse indicates a mammalian gametic doubling dose for specific locus mutations from gamma radiation of about 40 rad. Thousands of children have been born to parents exposed to the A-bomb who collectively have received on the order of 100 rad, corresponding to roughly a gametic doubling dose if mice and humans have similar radiation sensitivity. Such doses for individuals have been reasonably well defined, and the careful work of the Atomic Bomb Casualty Commission (ABCC) and now the Radiation Effects Research Foundation (RERF) has kept track of the individuals and their health effects (*1*).

Conventional methods to detect adverse reproductive or genetic health outcomes in the A-bomb offspring have yielded no results. Pregnancy outcome, childhood mortality, and sentinel phenotypes have been looked at to varying degree without any indication of a dose-response function or a significant difference between exposed and controls. (Note that this does not apply to the effects of *in utero* exposure where clear cut and low-dose effects have been observed.) An extensive search for induced heritable chromosome aberrations has also been negative.

Over the past 14 years, a major effort to find mutational change in gene products has been carried out by the RERF staff under the inspiration of James Neel (*1*). The RERF method uses gel electrophoresis to identify a charge change in enzymes that retain sufficient enzymological function to be identifiable on the gels. Some 30 enzymes in the red blood cells of tens of thousands of children of A-bomb survivors have been examined. When an unusual pattern is found in a child, the parents are tested both for the absence of the variant and for biological paternity. A mutation is scored when all three results are positive. As summarized in Table 1, only six mutations have been found in more than 1 million loci tested (*2*). These mutations divide equally into the exposed and control populations, indicating a background electromorphic mutation rate of 0.5×10^{-5} and no observable effect on mutation rate from the 100 rad exposure.

It is instructive to relate these results back to the mouse. The average gonadal dose these children's parents received is approximately the same as the mouse doubling dose, and hence by direct analogy one might have expected something like six mutations in the exposed group and three in the control group, rather than three mutations in each group. The absence of the dou-

Table 1. One-dimensional electrophoresis of children of A-bomb survivors, 1972–1986.[a]

	Proximally exposed parents	Distally exposed parents
Dose, T65		
Gamma, rad	74.8	< 1
Neutron, rad	10.9	
Loci tested	725,587	539,170
Mutations	3	3
Mutation rate per locus per generation	0.4×10^{-5}	0.5×10^{-5}

[a] From Satoh et al. (*2*).

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bling effect raises several possibilities: statistical fluctuation is secondary to the smallness of the numbers, the human is more resistant to mutation than the mouse, or something is wrong with the experiment. Perhaps the best explanation is the difference in the two end points being detected, i.e., the loss of function of one of seven recessive genes in the mouse versus a charge change in the presence of residual enzyme function in the human. The human end point is a relatively less likely outcome of ionizing radiation, and when the very approximate data on frequencies of types of background and radiation-induced DNA damage are factored into the human data, the results suggest an expected increase of electromorphs of something like 30% rather than 100% (*3*). This places the expectation in the human at four and three mutations in the exposed and control groups, respectively, instead of three mutations in each group. To detect an outcome of this magnitude at the 5% level of significance with a power of 90% would require the harvesting of over 500 mutations. According to this line of reasoning, the data fall close to expectation, but are 100 times too small.

Two important points about the demands on any mutation-detecting method should be made. First, the events are exceedingly rare, even for this dramatically exposed population; and second, one must have a very clear understanding of the types of events that are expected and that can be detected by one's method in order to anticipate the yield and to relate the results to other data sets or to risk.

The Potential for New Methods for Heritable Mutation

There are two classes of new methods for detecting heritable mutation: those involving gene products and those involving DNA. The one entry in the first class is a direct extension of one-dimensional electrophoresis to the second dimension (*4–6*). In two-dimensional electrophoresis, gene products (proteins) are characterized by charge and by mass. There is a gain of both physical information about the protein and number of protein types seen, but a loss of functional information about the proteins. Hundreds of spots may be seen in a two-dimensional gel, but few of the spots are collated to known molecules or to underlying function. In addition, it is difficult in current gels to estimate the amount of material in many spots on the same gel, and thus to know when half of a spot is missing, signaling the possibility that one of the two alleles is no longer represented in that spot. Although the matter is controversial, there is a serious question as to whether this method will provide the power necessary to become an effective screening test for heritable mutation.

The other class of methods involves DNA, or more specifically, deviations of the child's DNA from that of its parents (*3*). The dramatic explosion of DNA technology in the past decade has resulted in remarkable power to manipulate and analyze the genetic material. Four examples of how this might be used for mutation testing will be discussed briefly.

The best known and most widely used method is restriction-fragment-length-polymorphism (RFLP). Figure 1 illustrates how the combination of a restriction enzyme and a DNA probe define a DNA fragment of specific length. When either of the bounding restriction sites is eliminated by a mutational change in the DNA, there is a clear alteration in the fragment length. In fact, each combination of six-cutter restriction enzyme and DNA probe tests, in a haploid genome, for the integrity of 18 basepairs, 12 by potential loss of site, and 6 by potential gain of site. Similar effects can also be produced by DNA rearrangements which change the distance between bounding sites. The technology to demonstrate such effects is in wide use today, but its application to mutation testing is severely inhibited by several factors. The background rate for heritable base changes in the human genome is estimated to be 10^{-8}. Thus, it would be necessary to examine on the average 10^8 basepairs to find one background mutation. As an example, this number of basepairs could be tested by using 10 restriction enzymes, 278 probes, and the diploid genomes of 1,000 children. Since a typical DNA gel can hold about 30 lanes (from 10 parent-child triads) and can be used for 20 probes, such an RFLP, examines 7200 basepairs. The measurement of 10^8 basepairs requires 14,000 gels and costs approximately $1.4 million per single background event. Given these logistics, RFLP is not a practical candidate for mutation testing of base substitutions at this time, but it does serve as an abstract benchmark for comparing other methods.

The second DNA method to be considered is DNA sequencing. In a sense, sequencing is the ultimate method to analyze mutations, since the complete sequences of the triad of involved genomes contains all

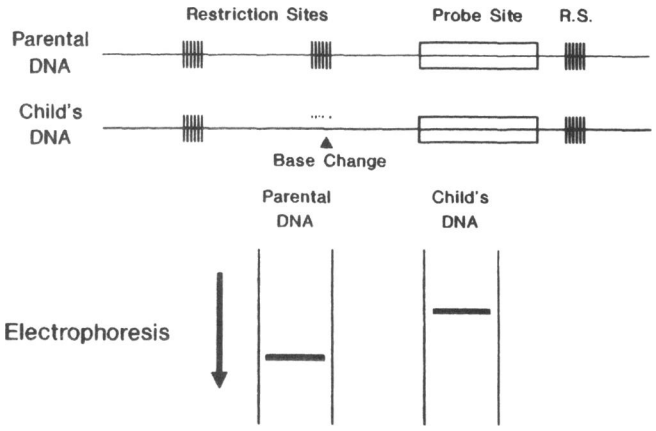

FIGURE 1. Restriction fragment length polymorphism applied to mutation detection. Parental and child's DNA are compared for probe site and six-cutter restriction site. In the child, the base change points to substitution of one of the six bases of the prior cutting site. This change neutralizes the site and results in the expansion of the DNA fragment to the next site. The difference in size is detectable in a Southern blot taken from a sizing gel.

the information to count and characterize mutational change. Perhaps some day such an approach can be considered, but to put this issue in perspective, one has only to observe the current controversy over the impact on biological science of sequencing just one human genome, let alone hundreds.

The third method takes advantage of the ability of RNAse A to cleave mismatches in RNA:DNA heteroduplexes (*7*). Probes of complementary, radiolabeled RNA are prepared from representative parts of a reference genome and are allowed to hybridize to conveniently sized pieces of genomic DNA from parents and child. Treatment with RNAse will cut the RNA at sites of mismatch and give a characteristic pattern when the RNA is run out on a sizing gel. Comparison of the patterns within the triad should indicate base changes and small rearrangements unique to the child. The method detects more that 50% of single base changes, and has an estimated efficiency that is more than 10 times greater than RFLP. Even with this improvement, the cost and effort make this very difficult as a practical application. At least three laboratories are exploring the future possibilities of the method.

The fourth DNA method to be considered is the least well understood from a practical viewpoint, but is worth considering here because of its exciting potential for enormous increases in efficiency. It is called subtractive hybridization and was introduced several years ago by George Church (*3*). It is based on the dramatic effect on melting temperature of a single base mismatch between an 18-mer of DNA and a fragment of genomic DNA in a solvent that minimizes adenine-thymine–guanine-cytosine differences in melting. To take advantage of this, Church visualizes a library of all possible 18-mers of DNA. The genomic DNA of both parents is combined, fragmented to 50 to 200 basepairs in size, mixed in large excess with an aliquot of the 18-mer library, and heated in tetramethyl ammonium chloride to just below the melting temperature. After sufficient time to maximize hybridization of perfect matches, the unmatched 18-mers are separated and mixed under the same conditions with the child's DNA. Since these 18-mers represent sequences that are not found in either parent, the presence of perfect matches with the child will signify mutations. In the final stage, such matches can be separated and analyzed by a variety of methods to count the events, find the involved genes, or specify the precise mutational changes. Since with the exception of the last stage, the analysis of the entire genome is done in parallel by Church's method, the relative efficiency can be enormous. The question is whether or not the thermodynamics of hybridization and the subsequent separations will permit the necessary discrimination to make the method work.

Of these four DNA methods, the RNAse cleavage approach is the only one that comes close to practical application at present. The hope is that further advances will be made, suggesting other parallel or otherwise highly efficient methods, and that the Church method will be properly evaluated.

A fifth type of DNA method is the application of DNA hybridization to cytogenetics. Chromosome painting by hybridization was first shown in human-hamster hybrid cells in which the human chromosomes were brilliantly stained using fluorescently labeled human genomic DNA (*8*). Translocations of the painted chromosomes are easy to recognize, and scoring them is orders of magnitude faster than conventional methods. Painting specific human chromosomes in human cells is under development and relies on the selection of batteries of unique sequence DNA probes from chromosome-specific gene libraries. Human chromosome 21 can already be partially painted with this method (*9*). An exciting variant of this method has the potential to extend cytogenetics into the microchromosomal region by combining the painting with developing methods of DNA manipulation. The manipulation is by orthogonal field alternating gradient electrophoresis (OFAGE), which for the first time permits resolution of large, million basepair or more DNA molecules (*10,11*). Cantor and colleagues have already demonstrated that an eight-cutter digest of human chromosome 21 produces a clear, reproducible series of bands in OFAGE (personal communication). The microcytogenetic potential of these two methods can be understood by imagining an eight-cutter digest of human genomic DNA. When run by OFAGE, such DNA will show a collage of overlapping bands coming from each of the 24 human chromosome types. By painting this preparation with DNA probes designed for chromosome 21, only the bands from chromosome 21 should light up, thus providing both the chromosome specificity and the subchromosomal resolution. This microcytogenetic display is two orders of magnitude below current methods and should have the power to see a representative sample of rearrangements in the size range between chromosomes and cosmids—currently a kind of no-man's land of genetic resolution.

Potential Methods for Germinal Mutation

As has already been pointed out, the rarity of heritable mutation greatly complicates the measurement of altered mutation rate in small numbers of exposed people. However, as with somatic mutation methods, the limited number of subjects can be sidestepped by focusing attention on sperm where samples of millions of cells are obtainable from a single male. The problem then becomes how to identify mutations when dealing with single cells. At the moment, suitable DNA methods at this level of resolution are not available, but there are good precedents for identifying mutated cells from their gene products.

Of these precedents, the prototype that may be extendable to germinal cells is the somatic mutation assay using glycophorin A (*12,13*). This sialoglycoprotein is the most abundant macromolecule in the human red cell membrane and is responsible for the M and N serotypes.

Half of the population is heterozygous MN, and in such people, the two alleles are codominant, putting 250,000 copies of each protein into every red cell membrane. The M and N forms differ by only two amino acids in the external, terminal region of the molecule. Monoclonal antibodies have been generated to mark each protein with high affinity and specificity. Using flow cytometry, one million red cells can be screened in 15 min, identifying and sorting all cells that have a normal amount of one allelic product and none of the other. These presumptive gene loss mutants occur at the rate of 10 per million red cells in normal heterozygotes, appear to increase linearly as a function of radiation dose in A-bomb survivors (*14*), and also respond to a variety of mutagenic cancer chemotherapies.

To conduct a comparable test with sperm requires similar markers that are controlled independently by two alleles or two related genes. In work in progress at this laboratory, Wyrobek and colleagues have chosen the two human protamines as a likely target. These molecules are exclusive to sperm, are expressed in the haploid state, and are tightly integrated into the nucleus with minimal chance for intercellular interaction. Monoclonal antibodies have been prepared to distinguish the two protamines (*15*), and very early pilot flow-cytometric experiments have shown control measurements and results comparable to glycophorin A. It is too early to claim this as a successful mutation method, but the principle is clear and should be applicable to other molecules.

The general requirements for such biomarkers of mutation fall into two categories. For positive biomarkers, one starts with a known mutant gene product, develops a method to identify it in single cells, and then searches directly for the mutant cells. An example of this approach using antibody detection is the search for sickle red cells in people who have not inherited a sickle gene (*16*). We have prepared good monoclonal antibodies for detection of sickle hemoglobin at the single cell level (*17*), and have learned how to apply them to red cells in suspension. Since the hemoglobin mutants represent a specific change of a specific base, they are expected to occur at two-orders of magnitude lower frequency than loss of gene product. Thus, it is not surprising that the sickle cells have apparently been found at rates approximating one per 10 million cells in normal blood. We have been unable to measure events of this rarity with sufficient reliability to mount a practical method. In addition, our experience with glycophorin A suggests another limitation based on the finite number of erythrocytic stem cells available to express the mutation rate. Our current data indicate that there are enough stem cells to carry the 10 in a million rate for gene loss, but at doses of several hundred rad, the measurements of mutant cells become discontinuous and must be averaged across subjects to be useful. We lack the data to be certain, but the hemoglobin system should be essentially nonresponsive in the single subject because of this limitation. It is likely that the same will be true for almost any system using a positive biomarker.

For negative biomarkers, the strategy is to find detection methods for normal gene products. In this case the problem is to control for the metabolic, physiologic, and other toxicologic reasons for nonexpression or nonmeasurement of the gene product. In the case of glycophorin A this is done by having the two codominant alleles separately labeled and insisting that one express normally. In the case of protamine, the approach is to use two genes with almost identical function, in the hope that what happens to one (other than mutation) happens to the other. For glycophorin A we are dealing with enucleate red cells and have no direct way as yet to confirm the mutational nature of what is being observed. In the case of the protamines there is the possibility to collect the mutant cells and use their DNA to try to confirm what has happened. In both cases the approximate frequency of expected events is known, and one can use the classical response to mutagens to add confidence that it is indeed mutations that are being measured.

Overview

It should be clear by now why this type of use of biomarkers is demanding and exceptional. It is hoped that within a few years it will also be clear that the approach is successful in this very important aspect of reproductive toxicology—the estimation of heritable and germinal mutation in human populations.

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